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Effects of prior aerobic exercise on high-fat meal-induced inflammation

Kelly Nicole Zender Fuller
Iowa State University

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**Effects of prior aerobic exercise on high-fat
meal-induced inflammation**

by

Kelly N. Z. Fuller

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Nutritional Sciences

Program of Study Committee:
Rudy J. Valentine, Co-Major Professor
Rick L. Sharp, Co-Major Professor
Marian L. Kohut
Matthew J. Rowling

Iowa State University

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LIST OF ABBREVIATIONS

ACC- Acetyl-CoA carboxylase
 AI – Augmentation index
 Akt – Protein kinase B
 AMPK – AMP activated protein kinase
 ANOVA – Analysis of variance
 AP-1 – Activator-protein 1
 AS160 – Akt substrate of 160 kDa
 AUC – Area under the curve
 BCA – Bicinchoninic acid assay
 BSA – Bovine serum albumin
 cAMP – Cyclic adenosine monophosphate
 CD11b – Cluster of differentiation molecule 11b
 CES-D – Center for Epidemiologic Studies Depression Scale
 CREBP – cAMP response element-binding protein
 CRP – C-reactive protein
 CVD – Cardiovascular disease
 DAG – Diacylglycerol
 DNA – Deoxyribonucleic acid
 ERK – Extracellular-signal-regulated kinase
 FFAs – Free fatty acids
 GLUT – Glucose transporter type
 GSK3 β -- Glycogen Synthase Kinase 3 β
 HbA1c – Glycated hemoglobin
 HDL – High-density lipoproteins
 HFD – High-fat diet
 HFM – High-fat meal
 HR – Heart rate
 HRP – Horseradish peroxidase
 ICAM-1 – Intercellular Adhesion Molecule-1
 IL-10 – Interleukin-10
 IL-18 – Interleukin-18
 IL-1 β – Interleukin 1 beta
 IL-6 – Interleukin-6
 IR – Insulin resistance
 IRS1 – Insulin receptor substrate 1
 JNK – c-Jun N-terminal kinase
 LDL – Low-density lipoproteins
 LPL – Lipoprotein lipase
 LPS – Lipopolysaccharide
 LPS – lipopolysaccharide
 MAPK – Mitogen-activated protein kinase
 MCP-1 – Monocyte Chemoattractant Protein-1
 MFI – Multidimensional Fatigue Inventory

mTOR – Mammalian target of rapamycin
mTOR – mammalian target of rapamycin
MUFA – Monounsaturated fatty acid
NF- κ B – Nuclear factor-kappa B
NF- κ B – Nuclear factor kappa B
NO – Nitric oxide
OGTT – Oral glucose tolerance test
PBMCs – Peripheral blood mononuclear cells
PBS – Phosphate buffered saline
PKC – Protein kinase C
PKD – Protein kinase D
PP – Pulse pressure
PPL – Postprandial lipemia
PSQI – Pittsburgh Sleep Quality Index
PSS – Perceived Stress Scale
PVDF – Polyvinylidene difluoride
qPCR – Quantitative polymerase chain reaction
RER – Respiratory exchange ratio
rFC – Recombinant factor C
RNA – Ribonucleic acid
ROS – Reactive oxygen species
RPE – Rate of perceived exertion
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM – Standard error of the mean
SEM – Standard error of the mean
SFA – Saturated fatty acid
SIRT1 – Sirtuin 1
T2D – Type 2 Diabetes
TBST – Tris-buffered saline with Tween
TG – Triglycerides
TGRL – Triglyceride-rich lipoprotein
TLR2 – Toll-like receptor 2
TLR4 – Toll-like receptor 4
TNF α – Tumor necrosis factor- α
VCAM-1 – Vascular cell adhesion molecule 1
VO₂ – Oxygen uptake
VO₂ max – Maximal oxygen uptake
W – Watts

ABSTRACT

Lipemia and systemic inflammation are involved in the development of metabolic disorders including atherosclerosis, Type 2 diabetes and metabolic syndrome. Additionally, peripheral blood mononuclear cells (PBMCs) have been shown to play a role in the development of these diseases through the production of pro-inflammatory cytokines and cell infiltration. Aerobic exercise has been shown to have an anti-inflammatory effect in circulation and also attenuates postprandial blood lipids, however the effect of exercise on postprandial inflammation in PBMCs remains unclear. The aim of this study was to determine the protective effect of a single bout of aerobic exercise against postprandial lipemia and inflammation in PBMCs and to evaluate whether these manifestations are associated with changes in the energy-sensing enzyme, AMP-activated protein kinase (AMPK) as an underlying mechanism.

Healthy male subjects ($n=12$, age= 23 ± 2 , %Fat= 19 ± 2) reported to the laboratory following an overnight fast (12-14h) on two separate occasions utilizing a randomized crossover design. The afternoon prior (16-18h) to one of the morning HFM visits participants completed an acute bout of aerobic exercise (45-min of cycling at 65% of VO_{2peak}). Subjects consumed the HFM (1,000kcal, 56%Fat) following a baseline blood draw and water was available *ad libitum* throughout all visits. Plasma triglycerides (TG) were quantified using an *in vitro* assay. PBMCs from fasting and 4hr postprandial blood were isolated using the Ficoll Paque Plus method, lysed and equal protein was separated by SDS-PAGE. Western blotting was used to assess AMPK and inflammatory signaling pathways. Statistical analysis was performed between the control and exercise sessions at pre- and post-meal time points using a two-way ANOVA.

Our results show that an acute aerobic exercise bout (65% of $\text{VO}_{2\text{peak}}$) increased PBMC AMPK activation demonstrated by increased ACC phosphorylation ($p=0.05$). Increased ERK phosphorylation ($p<0.01$) following the HFM reflected induced postprandial inflammation in PBMCs. The exercise intervention significantly decreased postprandial plasma TGs ($p=0.02$) and maintained pAMPK T172 signaling ($p=0.04$) compared to control. Markers of inflammation, such as expression of phosphorylated NF- κ B (p-NF- κ B), were also attenuated in PBMCs with prior exercise ($p=0.02$).

In conclusion, the HFM induced postprandial lipemia and inflammation in PBMCs. A single bout of 45mins of moderate aerobic exercise significantly reduced postprandial lipemia and attenuated fasting and postprandial measures of inflammation, including NF- κ B signaling. Ongoing analyses will further describe molecular changes involved in the regulation of AMPK signaling.

CHAPTER 1 – GENERAL INTRODUCTION

In recent years obesity has reached epidemic proportions across the United States as well as globally. There are many medical complications associated with obesity, among which insulin resistance, type 2 diabetes (T2D), dyslipidemia, cardiovascular disease (CVD) and inflammation are of large concern [1]. Despite the well documented prevalence of these and other comorbidities, the cellular mechanisms of how obesity and excess nutrient intake play a role in the development of these conditions remains unclear.

Due to Westernized diets and large meal proportions, humans spend the majority of the day in a postprandial state. Because of this, researchers have turned to the postprandial period to try to identify metabolic contributors to disease. In recent years it has been determined that consumption of a single high-fat meal (HFM) can lead to dyslipidemia and systemic inflammation [2]. Dyslipidemia is characterized by elevated plasma cholesterol, triglycerides (TG) and low-density lipoproteins (LDL) and a decrease in high-density lipoproteins (HDL; [3]). Postprandial dyslipidemia has been found to play a role in many health-related conditions such as atherosclerosis and cardiovascular disease [4].

Lipemia is defined as an abnormally high accumulation of lipid particles in the blood and it occurs regularly following consumption of a meal [5]. Exercise has been shown to have promising effects on improving postprandial lipemia as well as associated cardiovascular disease risk. This can transpire through decreases in postprandial glucose, triglycerides [6] and increases in insulin sensitivity [7]. Prior exercise successfully decreased postprandial TG independent of exercise type, timing of exercise, fat content of test meal, energy balance and disease status and age of participants [8]. The majority of the

literature to support this information was done in healthy male populations and with aerobic exercise completed the afternoon prior to consumption of a morning test meal.

Low-grade inflammation has been identified as an early contributor in the pathogenesis of many metabolic disorders including atherosclerosis, T2D, hypertension, metabolic syndrome and insulin resistance [9]. Due to the increased prevalence of these disorders, mild chronic inflammation is of research interest. Exercise and physical activity are known to have many anti-inflammatory effects and can acutely improve systemic inflammation seen in the postprandial state. Examples of this are increased antioxidant gene expression [10] and increased levels of circulating endothelial progenitor cells [11], both of which are anti-inflammatory. Additionally, aerobic exercise training has been shown to decrease multiple serum inflammatory mediators known to increase in relation to metabolic diseases. Specifically, C-reactive protein (CRP), Interleukin-6 (IL-6) and Interleukin-18 (IL-18) concentrations have been shown to decrease in response to an aerobic exercise training protocol [12].

Vascular outcomes have been widely used as a non-invasive assessment of disease risk and metabolic health. Peripheral and central blood pressures are commonly used as early disease indicators in healthy populations as well as in disease states. For decades studies have shown that obesity is related to hypertension and decreased vascular function[13]. There is also strong evidence that consumption of a HFM leads to elevated peripheral blood pressure in young, healthy individuals [14]. Flow-mediated dilation and microvascular blood flow have been shown to decrease in healthy populations during the postprandial state following a high fat meal challenge [15].

A number of key metabolic pathways have been recognized as links between excess caloric intake and disease. Notably, AMP-activated protein kinase (AMPK) has been identified as a regulator of cellular energy balance and its inhibition is tied to many metabolic disorders [16]. Activation of AMPK in peripheral tissues such as skeletal muscle, liver and adipose tissues increases glucose transport, fatty acid oxidation and mitochondrial function [17]. However, in response to high glucose, fatty acids, or branched chain amino acids, literature suggests that AMPK activity is down-regulated [18, 19]. This inhibition of AMPK leads to impaired insulin signaling and in the case of chronic nutrient excess, eventual insulin resistance [20]. Exercise significantly increased AMPK activation in many peripheral tissues related to metabolic diseases, most notably skeletal muscle, adipose tissue, and liver [21]. Caloric restriction and exercise are two known promoters of insulin sensitivity and AMPK activation, leading to the identification of AMPK as a potential drug target for T2D [22]. AMPK regulation with excess nutrients and exercise is not well understood in immune cells, specifically peripheral blood mononuclear cells (PBMCs), but may also play a role in the development of these and other inflammatory diseases.

The first aim of this research project was to 1) determine whether an acute bout of moderate intensity aerobic exercise increases AMPK phosphorylation in PBMCs. The second aim was to 2) determine if consumption of a high-fat meal can downregulate AMPK phosphorylation, after moderate intensity aerobic exercise. Lastly, this study aims to 3) evaluate whether 45mins of acute aerobic exercise is protective against postprandial inflammation. We hypothesize that the administered high-fat test meal will decrease AMPK activation and that prior exercise will defend against postprandial systemic and cellular

inflammation. The proposed pathways of interest from a HFM to inflammation and metabolic disease are depicted in Figure 1.1.

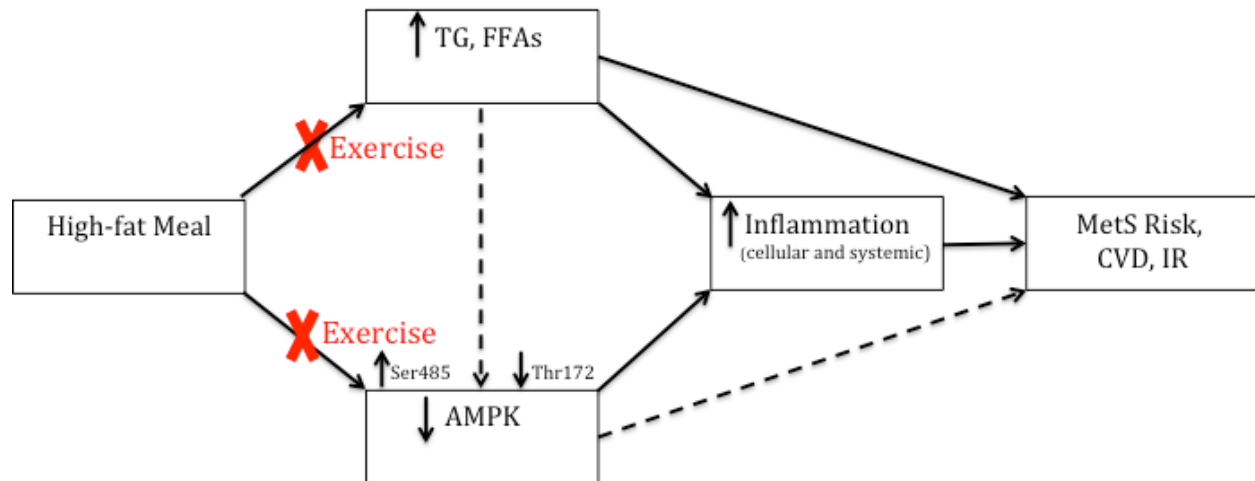


Figure 1.1 – Proposed pathway from HFM to inflammation and metabolic disease.

CHAPTER 2 – REVIEW OF LITERATURE

2.1 POSTPRANDIAL METABOLISM

Postprandial metabolism highly depends on the composition of the ingested meal, meal frequency and the duration of meal consumption and gastric emptying [23]. In healthy individuals it is expected that plasma glucose and insulin responses increase with relative carbohydrate composition and decrease with protein composition [24]. Following the intake of a HFM, the postprandial period is marked by a number of adverse metabolic responses. In today's society individuals are less active and regularly consume larger meals with a higher fat composition [25]. This trend leads to individuals spending more time in the postprandial state and thus experiencing chronic postprandial lipemia (PPL) and metabolic dysregulation. PPL is the rise in blood triglycerides following a meal and has been linked to obesity [26], increased atherosclerotic plaque formation [27], cardiovascular disease, metabolic disease and insulin resistance [4]. Increased PPL is also associated with impaired endothelial function and increased inflammation, two factors that are closely related to many cardiovascular and metabolic disorders [28].

The scientific and medical fields recognize postprandial measures as stronger assessments of disease risk compared to fasting measures [29]. Glycemic control is a high priority for individuals with T2D as well as other metabolic diseases. Glycated hemoglobin (HbA1c) is a regularly used indicator of long-term glycemic control, usually reflecting blood glucose levels over several months [30]. However, blood glucose is highly impacted by types and frequency of meals as well as by physical activity. Due to this, it is important to measure glucose responses throughout the day and continuous glucose monitoring has

recently provided additional research insight into the pathology of metabolic disease. Because postprandial glucose assessments capture meal-induced changes they are considered more tightly related to disease risk and are a target outcome for management and prevention strategies [31].

Several measures of interest have been identified when evaluating postprandial metabolism and lipemia. A blood lipid profile is commonly run, which often includes measures of plasma TG, total, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol. Plasma FFAs are also regularly quantified to assess what changes may be occurring in lipid metabolism. Glucose and insulin are measured following meal tolerance tests to assess glycemic response and insulin sensitivity, as the above measures closely correlate to disease states.

Postprandial metabolism is strongly affected by meal composition. Surina and Langhans studied the differences in postprandial fatty acid oxidation following consumption of equal caloric high carbohydrate and high fat meals. The fat content of the HFM was able to acutely affect fatty acid oxidation, indicated by a marked increase in plasma free fatty acids (FFAs), plasma TG levels and beta-hydroxybutyrate compared to the high carbohydrate meal [32]. Type of fat (monounsaturated or saturated fat) also appears to impact postprandial metabolism. When measured in healthy subjects, dyslipidemic postprandial insulin responses are less pronounced as the proportion of fat from saturated fatty acids (SFAs) increases. This same result was shown in subjects with high fasting TG levels, resulting in lower lipemic and insulinemic responses [33]. When consuming a meal composed of monounsaturated fatty acids (MUFAs) compared to SFAs, insulin resistance during the postprandial period was attenuated [34].

2.2 EFFECTS OF EXERCISE ON POSTPRANDIAL LIPEMIA

Substrate kinetics during the post-exercise postprandial period have received increased research attention in large part due to evidence that suggests that prior exercise can serve as a protective mechanism against postprandial lipemia. Moderate intensity exercise 16-20 hours before consumption of a meal lowers the post-meal response of plasma TG [6, 35], total cholesterol and LDL apolipoprotein B concentrations [36]. A single bout of aerobic exercise has also been shown to decrease postprandial glucose responses and increase insulin sensitivity for as long as several days [7]. The majority of research has been done on aerobic exercise, however resistance exercise has also been shown to attenuate postprandial hyperlipemia linked to increased risk of cardiovascular disease and metabolic syndrome [37].

The mechanisms underlying the postprandial effect of exercise are not fully elucidated. One potential mechanism is that increased TG hydrolysis by stimulated lipoprotein lipase (LPL) activity, a key enzyme in plasma triglyceride catabolism [38], are responsible for the improved postprandial lipid status following prior exercise. However, the literature reflects mixed results on whether or not LPL activity is altered, as it has increased postprandially with prior exercise in some [39] but not all [36] studies. In general the LPL response is related to the energy expenditure associated with the exercise bout, with higher expenditure related to increased enzymatic activity [40]. It is important to note that attenuated postprandial lipemia can be achieved in the absence of increased LPL activity [36].

Other suggested explanations for the postprandial effects of an acute bout of exercise include the consequences of changed energy homeostasis. As energy expenditure

during exercise increases, corresponding postprandial lipemia decreases, as marked by lower plasma lipid molecules [8]. Similarly, when the energy deficit was replaced following exercise, there was less of a pronounced effect on post-prandial metabolism [41]. Despite low energy expenditure, an acute bout of resistance exercise was able to attenuate a postprandial lipemic response similar to those seen in other aerobic exercise protocols with double the energy expenditure [37]. This serves as evidence that the effect of resistance exercise effect may instead be due to factors related to vigorous muscle contraction. When assessing the effects of exercise training, adaptations such as improved body composition, increased muscle GLUT-4 protein concentration [42] and increased skeletal muscle capillary density [43] have been shown to play a role in postprandial adaptations.

A quantitative review by Freese, Gist, and Cureton pointed out that exercise performed prior to a meal caused a moderate reduction in TG regardless of exercise type, timing of exercise, fat content of test meal, energy balance, and disease status and age of participants [8]. Reduced lipemia with exercise has regularly been found in both healthy individuals [44, 45] and those with metabolic related diseases [46, 47], however a number of studies have suggested that healthy individuals may require a higher intensity exercise stimulus to elicit a glycemic response. For example, Numao et al. studied the effects of a single bout of aerobic exercise performed immediately prior to an oral glucose tolerance test (OGTT) at 50% VO_2 peak during the postprandial period following a 3-day low-carbohydrate, high-fat diet (HFD; [48]). The described methods resulted in no improvement in postprandial plasma glucose or insulin sensitivity with exercise following the HFD. The lack of response may be attributed to a low intensity exercise and/or a multi-

day HFM intervention. Alternatively, Oberlin et al. assessed the effects of postprandial glycemia in individuals with T2D and found that a single bout of aerobic exercise performed at 60-75% heart rate reserve significantly lowered 24hr postprandial plasma glucose concentrations [46].

Although some studies mentioned above show a multi-day effect of exercise on postprandial lipemia, the majority of the studies show that exercise effects are rather transient in nature. Due to the short-lived improvements on postprandial TG, FFAs, insulin and glucose, data suggests that exercise should be performed daily in order to experience the optimal effects [36].

2.3 HIGH-FAT MEAL-INDUCED INFLAMMATION

The rate and magnitude of systemic inflammation following a meal has been associated with meal composition; and lipids have been shown to increase pro-inflammatory cytokine production and innate immune cell activity significantly more than glucose in healthy, obese and T2D populations [2]. A five-week intervention study illustrated that dietary fatty acids modulate and increase plasma pro-inflammatory markers IL-6, CRP, and fibrinogen in comparison with a high-carbohydrate diet [49]. Similarly, an acute HFM (760 Kcal, 59% Fat) increased plasma TNF α , IL-6, ICAM-1 and VCAM-1 two hours postprandial in greater magnitude and duration than an isoenergetic carbohydrate meal [50].

Several inflammatory mechanisms have been identified in association with PPL and disease progression. In general, increased plasma lipids following ingestion of a meal accumulate in the blood and enter the vessel wall, stimulating a downstream pro-inflammatory cascade eventually resulting in atherosclerotic plaque formation [51].

Atherosclerosis is a multifactorial inflammatory disease caused in large part by chronic exposure to increased TGs. Recently a complex series of mechanisms have been identified in the progression of the disease, starting with HFM-induced PPL. Elevated TGs have been found to increase monocyte and neutrophil activation as well as contribute to pro-inflammatory endothelial cell signaling and the oxidative stress response [52]. These results have occurred in both healthy populations and those with risk of developing atherosclerosis [53].

During prolonged PPL, chylomicron remnants accumulate and LDL concentrations increase. In an *ex-vivo* experiment analyzing the effects of a HFM, it was found that increased density of TG, cholesterol, apolipoprotein C-III (apoC-III) and apolipoprotein E (apoE) correlated with increased atherogenic potential [54]. Plasma remnant lipoproteins are major pathological factors in several cardiovascular events, as they accumulate and play a role in the infiltration of mononuclear leukocytes [55]. Small, dense LDL particles characteristic of PPL are known to enter the vessel wall easily and bind strongly. The particles are then harder to clear from the arterial wall and become prone to oxidation, two factors making them more atherogenic than their large LDL counterparts [51, 56].

Postprandial endothelial dysfunction is linked to atherosclerosis and adverse cardiovascular events both directly and indirectly. When working in an optimal manner, the endothelium regulates the movement of cells, proteins, and other species across the luminal side of blood vessels. Endothelial cells are sensitive to a wide array of paracrine, autocrine and endocrine signals [56]. Through these signals, the endothelium is able to regulate many different physiological processes, which includes inflammation. Dysfunction of the endothelium has been tied to increased inflammatory cytokines and oxidative stress,

which can occur with chronic TG and LDL exposure [57]. Additional contributing factors to endothelial dysfunction are reactive oxygen species (ROS) and increased neutrophil remnants with augmented pro-inflammatory cytokines [58]. Once the vascular endothelium is abnormally regulated, monocytes are activated and differentiate into macrophages at the site of the arterial wall [56].

Pro-inflammatory cytokines, most notably IL-8 and MCP-1 are responsible for changing the morphology of monocytes. However, the most common pro-inflammatory factor that defines a proatherogenic state is the net up-regulation of VCAM-1 expression, that is independent of the stimulation of tumor necrosis factor- α (TNF α). Further information on the cell signaling mechanisms are discussed in sections to follow. CD11b is a transmembrane receptor that regulates the adhesion process through molecules such as ICAM-1 and VCAM-1. These mechanisms along with increased ROS activate monocytes and contribute to macrophage foam cell formation that leads to atherosclerotic plaque development [56].

Peripheral blood monocytic endothelial cell progenitors integrate into the vasculature *in vivo*. Among endothelial cell progenitors, PBMCs have been found to accurately reflect an inflammatory state. Circulating PBMCs, such as CD34+ primitive progenitor cells and CD14+ monocytic progenitors are easily harvested for study purposes and are generally thought to promote healing, blood flow restoration and vascular growth [59]. Isolation of PBMCs have previously been used to assess inflammatory responses to HFDs. For example, consumption of a one week HFD impaired toll-like receptor 2 (TLR2) expression in healthy populations [60]. Toll-like receptors are known for their involvement in mediating the production of cytokines. Additionally, impaired glucose responses to a

short-term HFD have also been linked to decreased AMPK activity and inflammatory mitogen-activated protein kinases (MAPKs) in PBMCs [61]. These findings further support that the short-term intake of a HFD has several immune system and inflammatory related responses.

NF- κ B is widely recognized as the classic inflammatory signaling pathway due to its role as a transcription factor for other pro-inflammatory cytokines, chemokines and adhesion molecules [62]. Activation of NF- κ B is accomplished through many post-translational modifications such as phosphorylation [63] and to carry out its function, the protein must enter the nucleus for access to transcription machinery. One widely studied inhibitory protein, I κ B α , functions to keep NF- κ B in the cytoplasm, thereby preventing its activation and the transcription of pro-inflammatory cytokines. However, once I κ B α is phosphorylated, it dissociates with NF- κ B, allowing it to translocate to the nucleus [64]. Postprandial decreases in I κ B α and increases in NF- κ B have been previously reported in mononuclear cells and may play a role in inflammation seen in obesity and metabolic disease [65].

Insulin resistance is a common medical complication of obesity and T2D and its pathogenesis by chronic excess nutrient exposure is linked to high-fat meal-induced inflammation. Pro-inflammatory cytokines can directly induce insulin resistance in multiple tissues [66]. However, the role of PBMCs in insulin resistance susceptibility to IR remains unclear. Skeletal muscle accounts for up to 80% of the total insulin-stimulated glucose uptake and therefore should be considered an important factor in the development of T2D [22]. Peripheral tissues and plasma of individuals who are insulin resistant reflect an increased level of oxidative stress, DNA damage and inflammation [17]. One proposed

mechanism for this is through the AMPK signaling pathway since AMPK is a major energy regulator that plays a key role in stimulating glucose uptake and the insulin-sensitive glucose transporter type (GLUT) 4 translocation [22].

2.4 EXERCISE AND THE INFLAMMATORY RESPONSE

Regular physical activity is largely recognized for having positive impacts on the immune system and has been found to decrease low-grade inflammation [67]. Examples of specific effects of aerobic exercise training include decreased concentrations of Interleukin-6 (IL-6), Interleukin-18 (IL-18; [12]), MCP-1, TNF α [68] and CRP [69]. However, recent studies have also shown that acute exercise may play an important role in protecting against many of the inflammatory disease progressions mentioned above.

Both acute and chronic aerobic exercise may reduce free radical formation in circulating PBMCs. Overall, endurance training was shown to promote blood vessel growth and inhibit inflammation independently from other confounding variables [70]. Acute exercise increases anti-oxidant gene expression in healthy populations, and in contrast, the sedentary state is associated with increased oxidative stress and superoxide levels. Elevated superoxide and decreased nitric oxide production is linked to cardiovascular and metabolic diseases through endothelial dysfunction [11, 71]. Therefore, the observed increase in nitric oxide production following an acute bout of aerobic exercise is evidence for beneficial responses with physical activity.

One bout of acute exercise has been shown to transiently increase the levels of inflammatory mediators throughout the body. Circulating levels of IL-6 and TNF α increase, in turn leading to an increase in other anti-inflammatory factors such as IL-10 and IL-1ra [72]. Skeletal muscle and adipose tissue are largely known for cytokine secretion, but more

recently PBMCs have been recognized for mediating inflammatory response during exercise. In a study by Ulven et al., serum levels of CXCL16, sTNFRI, IL-6, IL-10, TNF α , sICAM-1 and sVCAM-1 and PBMC mRNA levels of CXCL16, IL-1 β , IL-8, COX-2, GATA3 and TXB21 were found to increase immediately following a one hour cycling exercise bout completed at 70% of VO₂ max [73]. Pro-inflammatory factors IL-6 and TNF α increased with exercise in the serum but were not changed at the mRNA level, suggesting that the cytokines may be produced locally in response to muscle damage. Gene expression of IL-1 β , a potent pro-inflammatory cytokine and associated COX-2 both increased in PBMCs [73]. This is in contrast to works of others who demonstrated decreased or unchanged IL-1 β levels with exercise [74]. Differences may be dependent upon type and intensity of exercise, training status, or processing.

Gjevestad et al., published a recent review on the impact of exercise on PBMC gene expression of inflammatory markers [75]. Many of the studies included in the review provide evidence that exercise impacts both the innate and adaptive immune system through signaling molecules and transcription factors. The review highlighted strong evidence that exercise causes immediate increases in IL-6, TNF α , TLR2 [75] and TLR4 [76] among others. Alternatively, when individuals partake in regular exercise training, there is blunted IL-6 [77] and TNF α responses to an acute exercise bout [76]. Physical activity level, age and gender were shown to alter immune response with exercise due to changes in basal inflammatory profiles [78]. Due to these impacts, along with variation in exercise intensity and duration, many studies found no change in inflammatory markers with exercise [74]. Overall, regular physical activity further increases the anti-inflammatory effects seen with an acute bout of exercise and decreases the pro-inflammatory response

[75]. These immune responses are thought to play a role in decreasing the risk of atherosclerosis, T2D and other inflammatory diseases.

2.5 CELL SIGNALING MECHANISMS

As evidenced above, excess caloric intake and postprandial TG and FFAs are closely related to many cardiovascular and metabolic diseases. The cellular mechanisms responsible for the development of the aforementioned diseases are of great scientific interest as their identification and understanding may contain information pertaining to future drug targets. One such mechanism, and the main cellular pathway of interest for this study, is the energy sensor AMP activated protein kinase. AMPK is a master regulator of energy metabolism and works to maintain energy homeostasis [16]. AMPK is a serine/threonine kinase that is activated in low cellular energy conditions and is down-regulated in cases of nutrient excess [20]. Once activated, AMPK affects many tissues, including skeletal and cardiac muscle, liver and adipose tissue (Refer to Figure 2.1).

Activation of AMPK occurs when the intercellular AMP: ATP ratio is elevated and the Threonine 172 α -subunit is phosphorylated by an upstream kinase [22]. Once activated, AMPK phosphorylates many downstream molecules, resulting in the conservation of ATP and the activation of ATP-producing pathways [79]. In turn, AMPK serves as a protective mediator against several stress conditions within the cell, including oxidative stress, exercise and starvation [80]. Additionally, noted physiological impacts of AMPK activation include increased glucose uptake and fatty acid oxidation in muscle and liver and decreased insulin secretion. Exercise-stimulated glucose transport may be dependent on the activation of AMPK and may occur through the phosphorylation and activation of Akt substrate of 160 kDa (AS160; [81]). The above effects improve insulin sensitivity and

glucose homeostasis, making AMPK an attractive therapeutic target for metabolic diseases [22].

Importantly, exercise has been identified as one of the strongest physiological activators of AMPK signaling [82]. The phosphorylation of AMPK^{T172} activator site has been found to increase with exercise in the vast majority of the tissues studied [82, 83]. AMPK activity significantly increased in rat liver, muscle, and adipose tissue 30mins after an acute exercise bout compared to sedentary counterparts [21]. Activity of both isoforms increased, with liver responding predominantly to the α_1 subunit and muscle to the α_2 subunit. Similarly, we have previously found that a moderate intensity acute bout of treadmill running increased AMPK^{T172} phosphorylation in mouse adipose and liver tissue independent of dietary factors (low-fat or high-fat diet; Fuller and Valentine unpublished). Increased AMPK expression has also been noted in mouse aorta endothelial cells following acute exercise [84]. Moir et al. found that acute exercise (45mins, 70% VO₂ max) decreased AMPK activation in PBMCs [85]. Despite conflicting evidence in PBMCs, increased AMPK activation remains a promising target for improving the inflammatory response and metabolic state of individuals while decreasing the risk of developing metabolic disease.

Due to the extensive positive health implications of AMPK activation, its downregulation is of concern and has been shown to be reduced in cases of obesity and metabolic syndrome [86]. Nutrient excess (by high glucose, amino acids and fatty acids) impairs AMPK activation in multiple tissues and contributes to the development of insulin resistance caused by hyperinsulinemia [18, 87]. AMPK activity has been reported to be lower in insulin resistant morbidly obese individuals compared to their insulin sensitive counterparts [86]. In addition, insulin resistant individuals with down-regulated AMPK

activity in adipocytes experienced higher concentrations of pro-inflammatory genes in a tissue-specific manner [86]. Accompanied inflammation and oxidative stress lead to further harmful changes and disease progression, making AMPK a favorable activation target for preventing chronic insulin resistance [20]. Importantly, Wan et al. showed that a one-week high-fat diet caused AMPK down-regulation in PBMCs of healthy males [61].

One mechanism underlying the reduction of AMPK is the recently identified inhibitory phosphorylation site on the α -subunit, Serine 485/491. Akt and members of the protein kinase C (PKC) family of kinases have been shown to phosphorylate the Serine 485/491 site [20]. Although the mechanisms are not fully understood, PKC has been implicated as a major player in AMPK regulation and the development of insulin resistance [20]. Increased FFAs lead to elevated phospholipid diacylglycerol (DAG) concentrations, which in turn activate several PKCs [20]. This initial event then leads to decreased insulin signaling by blocking and inhibiting the insulin signaling cascade through the down-regulation of AMPK, AS160, insulin receptor substrate 1 (IRS1) and Akt [20].

PKCs have also been shown to directly activate mechanistic target of rapamycin (mTOR), an additional contributor to insulin resistance [88]. mTOR coordinates cellular growth in response to nutrient status and may play a role in activation of endoplasmic reticulum stress and lipid accumulation with nutrient overload. When phosphorylated and activated, AMPK has been shown to inhibit mTOR, preventing hepatic lipid accumulation and insulin resistance [80].

AMPK's influence is widespread beyond metabolic homeostasis, as it has been shown to play a role in cellular remodeling and decreased inflammation following a high-fat and high-glucose meal [89]. AMPK has most recently been recognized for playing an

anti-inflammatory role in immune cells, most notably for decreasing macrophage inflammation that can eventually lead to atherosclerosis. Yang et al. found that activating AMPK in the presence of many inflammatory stimuli, such as exposure to LPS, FFAs, and diet-induced obesity, induced a protective effect against macrophage inflammation and insulin resistance [90]. On the other hand, inhibition of AMPK expression by macrophage RNA results in significant increases in TNF α and IL-6 [91].

One proposed explanation for these effects is through the signaling mechanisms of Sirtuin 1 (SIRT1), another energy sensor molecule that also responds to inflammatory stimuli [90]. Interleukin-10 (IL-10), a powerful anti-inflammatory cytokine that helps control inflammatory disease progression in models of atherosclerosis, is regulated by AMPK signaling in mouse macrophages [92]. This was first shown by demonstrating that AMPK is required for isolated bone marrow macrophage cells to express IL-10. Once induced, IL-10 leads to increased phosphorylation of Akt. Additional evidence is that IL-10 activation is blocked when substrates upstream of AMPK were inhibited [92].

The NF- κ B pathway is one proposed signaling mechanism for how TG-rich lipoproteins modulate the inflammatory cytokine response postprandially. It is thought that postprandial TG-rich lipoproteins increase the expression and binding activity of nuclear factor- κ B (NF- κ B), activator-protein 1 (AP-1), and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREBP). Once bound to the promoters of inflammatory genes, triglyceride-rich lipoprotein (TGRL) VCAM-1 expression was increased and lead to monocyte recruitment [93]. Abdominal obesity was identified as a strong indicator of VCAM-1 expression, linking commonly applied epidemiological measures of disease risk (TG levels, waist circumference) with acute molecular markers of

endothelial inflammation [93]. AMPK is a key regulator of nitric oxide (NO) production that functions to inhibit leukocyte activation in endothelial cells and when activated, AMPK inhibits TNF α -stimulated monocyte adhesion by reducing expression of VCAM-1 [94].

It is important to note the role of AMPK in this sub-population of cells. T cells are major players in the immune response and make up a large portion of the PBMC cell population. AMPK has been found to regulate T cell metabolic adaptation and homeostasis [95]. More specifically, AMPK plays a crucial role in mRNA translation regulation and mitochondrial metabolism [95]. Also, the role of AMPK in fatty acid synthesis and oxidation has been noted as an essential part of T cell differentiation and subsequent immune cell function [96].

2.6 VASCULAR OUTCOMES

Vascular outcomes such as central and peripheral blood pressures and augmentation index are altered in the postprandial state and have been shown to be an indicator of disease risk and a tool for predicting future adverse cardiovascular events. It is well understood that aerobic exercise has a positive effect on cardiovascular health and physical activity can be used as a strategy for decreasing disease risk. Decreased vascular function due to a sedentary lifestyle or consumption of a high-fat diet has been shown to relate to insulin resistance and endothelial dysfunction, two major contributors to many metabolic diseases. The majority of the literature focuses on populations with metabolic or cardiovascular diseases, leaving many questions regarding healthy populations and early indicators of disease unanswered.

Due to increased prevalence of cardiovascular-related diseases, non-invasive markers of vascular function are of interest. Central blood pressure measurements are now

being recognized as stronger indicators of future cardiovascular events than peripheral blood pressures [97]. When compared to carotid artery hypertrophy, atherosclerosis and arterial stiffness, central pressure was found to be a better predictor of adverse clinical outcomes [97]. Inclusion of the use of pulse pressure (PP) as a vascular outcome has been shown to contribute information to predicting disease risk across many different populations [98] .

Consumption of a high-fat meal has been shown to decrease vascular function 4-hours postprandially. For example, two high-fat breakfast meals, one rich in SFAs and one rich in MUFAs, were used to observe postprandial blood pressure, arterial stiffness and augmentation index (AI). Augmentation index is an indirect measure of wave reflection and arterial stiffness and is calculated in terms of a ratio of pulse pressure using pressure wave and systolic pressure [99]. Increased AI is an independent risk factor for cardiovascular disease [100]. Both high-fat meals induced a blood pressure dependent decrease in AI in healthy males, but the change was not statistically significant when controlled for increases in HR [14].

Fasted blood pressure and insulin sensitivity can impact how a single high-fat meal effects vascular compliance in young healthy individuals. In a study by Blendea and colleagues, elevated fasting and peak serum TG were highly correlated with decreased large and small arterial compliance following consumption of a high-fat meal [101]. Participants classified as fat reactors (higher fasted serum TG, glucose and insulin) experienced significant increases in peripheral blood pressure following the high-fat meal. Typically increased vasodilation occurs with meal ingestion in response to postprandial insulin secretion, however this response did not occur in fat reactors. Vessel elasticity was

measured by diastolic arterial pulse waveform and did not change following consumption of a low-fat meal [101].

Recent evidence from Esser et al. suggests that pairing vascular outcomes with markers of inflammation may provide further explanation of the postprandial responses following a high-fat meal challenge. Postprandial reductions in flow mediated dilation and augmentation index were found with ingestion of a both high-fat breakfast and a standard breakfast. However, the high-fat meal produced a significant treatment effect on both central and brachial systolic blood pressures, corresponding to increased IL-8 serum and PBMC mRNA levels. This study helped identify that although no differences were found among typical markers of endothelial function (IL-1 β , IL-6, TNF α), the combined increases in blood pressure and IL-8 may signal an adverse postprandial endothelium environment [15].

An acute bout of exercise, like chronic training, has been shown to positively impact vascular function. In healthy subjects, a single bout of cycling exercise performed at 65-75% heart rate reserve significantly reduced pulsatile components of both central and peripheral arterial pressures. Aortic pulse pressure was also significantly reduced with exercise, in a manner that was correlated with changes in aortic pulse wave velocity [102]. These findings suggest that if done regularly, aerobic exercise could be used a tool to increase vascular function by attenuating aortic pulse pressure through a possible mechanism of decreased pulse wave velocity.

A few studies have extended the positive vascular impacts of exercise to the postprandial period and have found promising evidence for maintenance of vascular integrity. Acute aerobic exercise has been shown to improve vascular measures of

endothelial function both 16-18 hours prior to consumption of a high-fat meal as well as two hours following ingestion. One 90-minute walking bout at 50% VO_2 max improved next-day postprandial endothelium dependent and independent vascular function in both lean and obese individuals following ingestion of a high-fat meal [103]. These findings were demonstrated using microcirculatory techniques to quantify vasodilator responses. One 45-minute session of treadmill walking completed two hours after consumption significantly decreased 4-hour postprandial flow mediated dilation previously elevated due to meal consumption alone [104]. Similarly, Augustine et al. studied the effects of a single bout of resistance exercise on postprandial vascular function. Consumption of the high-fat meal increased arterial stiffness, which supports other findings mentioned above [105]. However, when participants completed a single bout of resistance exercise, peripheral arterial stiffness response improved without any adverse effects on central arterial stiffness [105].

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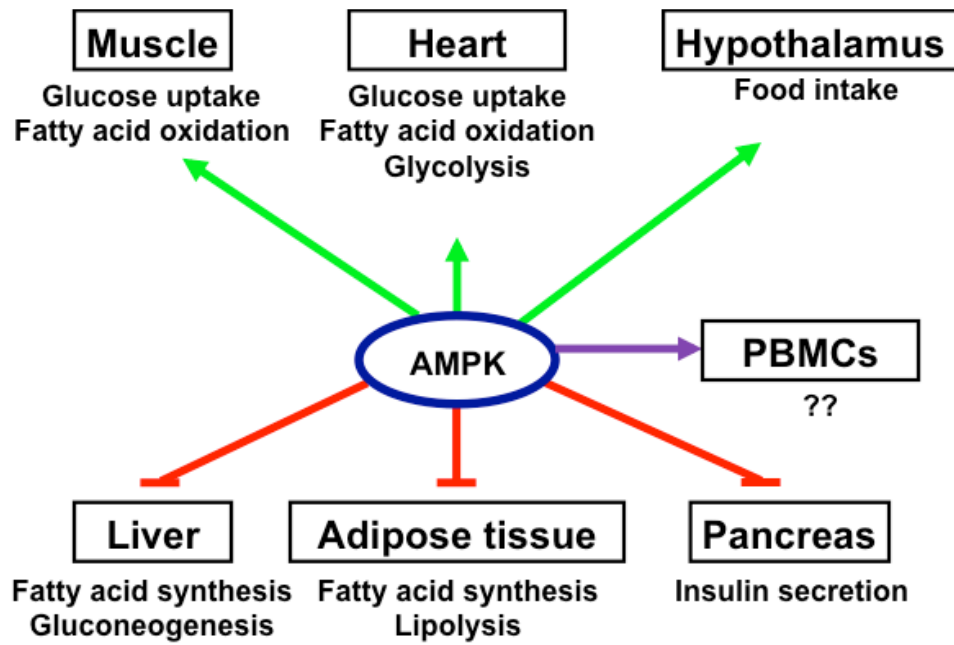


Figure 2.1: Roles of AMPK in the control of whole-body energy metabolism. (Adapted from Coughlan KA, 2014)

CHAPTER 3 – THE EFFECTS OF PRIOR ACUTE AEROBIC EXERCISE ON HIGH-FAT MEAL-INDUCED INFLAMMATION

Kelly N. Z. Fuller^{1,2}, Corey M. Summers², Rudy J. Valentine²

¹. The Interdepartmental Graduate Program in Nutritional Sciences, Iowa State University, Ames, IA. ². Department of Kinesiology, Iowa State University, Ames, IA.

Author contributions: KF and RV were responsible for the conception and design of the study and the interpretation of the data. KF, RV and CS completed data collection, while KF was responsible for data analysis and drafting the manuscript. All authors approved the final version of the manuscript.

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ABSTRACT

Chronic low-grade inflammation is involved in the development of metabolic disorders including atherosclerosis, Type 2 diabetes (T2D) and metabolic syndrome. Aerobic exercise has been shown to be anti-inflammatory and attenuate postprandial blood lipids, however the effect of exercise on postprandial inflammation remains unclear. The aim of this study was to determine the protective effect of a single bout of aerobic exercise against postprandial lipemia and peripheral blood mononuclear cell (PBMC) inflammation and to evaluate associations with changes in the energy-sensing enzyme, AMP-activated protein kinase (AMPK). Healthy male subjects (n=12, age=23±2, %Fat=19±2) reported to the laboratory following an overnight fast (12-14h) on two separate occasions for consumption of a high-fat meal (HFM). Participants completed an acute bout of aerobic exercise the afternoon prior to one of the HFM visits. Results indicate that the single bout of moderate aerobic exercise increased AMPK activation in PBMCs as shown by increased p-ACC. This may be due to decreases in the AMPK inhibitory kinases PKD and GSK3β.

Additionally, prior moderate intensity exercise decreased postprandial lipemia (PPL) and some mediators of the inflammatory pathway, such as NF- κ B.

3.1 INTRODUCTION

With the increased trend of Westernized diets, larger portion sizes and energy-rich meals, individuals spend the majority of their day in a postprandial state. Recent research has focused on the postprandial period rather than fasted measures because postprandial assessments are stronger predictors of disease risk and are a target outcome for disease management and prevention [1]. Following consumption of a meal, lipid particles accumulate in the blood, and if this occurs at an abnormally high level it is termed lipemia. Consumption of a single high-fat meal (HFM) has been shown to induce both lipemia and dyslipidemia, which is elevated plasma cholesterol, triglycerides (TG) and low-density lipoproteins (LDL) and a decrease in high-density lipoproteins (HDL,[2]). These conditions increase risk of adverse health conditions [3] and lead to increased systemic inflammation [4].

Emerging evidence suggests that acute exercise may improve postprandial inflammation. Independent of exercise type, timing of exercise, fat content of test meal, energy balance and disease status and age of participants, prior exercise decreases postprandial TGs [5]. This is important to note as lipemia has been linked to inflammation and atherosclerosis through the activation of leukocytes [6]. In addition, acute aerobic exercise performed prior to consumption of a high-fat meal attenuates postprandial intracellular ROS in PBMCs [7] and increased circulating pro-inflammatory cytokines such as IL-6 [8].

Though the evidence of improved postprandial inflammation with chronic exercise is strong, the effects and underlying mechanisms of acute exercise on postprandial inflammation remain unclear. Recently, AMP-activated protein kinase (AMPK) has been recognized for having anti-inflammatory properties [9]. AMPK is a whole-body energy sensor that is activated in states of low cellular energy (e.g. exercise, caloric restriction) and down-regulated with nutrient excess from both glucose and free fatty acids (FFAs) [10]. Once activated, AMPK initiates ATP-producing pathways, notably fatty acid oxidation and glucose uptake in skeletal muscle and inhibits anabolic processes such as fatty acid synthesis in peripheral tissues such as skeletal muscle, liver and adipose tissue [11]. The down-regulation of this enzyme by nutrient excess or hyperinsulinemia contributes to the pathogenesis of insulin resistance and other metabolic diseases [12, 13] and its activation may be a key component in the cellular mechanisms behind exercise-induced protection against low-grade inflammation.

Recent evidence has identified peripheral blood mononuclear cells (PBMCs) as reliable markers of postprandial inflammation [14]. These circulating immune cells can be used as an assessment tool for detecting inflammatory-related metabolic diseases in humans and correlate with inflammatory cytokines levels in the periphery [15]. Because of these reasons, and their direct role in the hyperinflammation seen with a HFM, PBMCs have previously been used to assess inflammatory responses to diet manipulation [16]. Additionally, AMPK activation has been shown to play an important role in the metabolic programming of T cells [17], a large sub-population of PBMCs. More specifically, AMPK has been shown to regulate T cell fatty acid oxidation and synthesis, related to cell

development and mitochondrial function, related to T cell longevity [18]. However, little is known about the regulation of AMPK in PBMCs.

This project aimed to address the aforementioned knowledge gap in the molecular mechanisms underlying the role of acute exercise on postprandial inflammation in PBMCs. In doing so, the present study investigated prior aerobic exercise as a preventative strategy against reductions in AMPK phosphorylation in PBMCs caused by a HFM, and to evaluate whether exercise is protective against postprandial lipemia and inflammation. It was hypothesized that the HFM meal would decrease AMPK activation in PBMCs and that prior exercise defended against postprandial inflammation through maintained AMPK signaling.

3.2 MATERIALS AND METHODS

Experimental protocol and ethics statement

Participants reported to the laboratory on four separate occasions to complete the experimental procedure. All volunteers completed identical first visits, but the study utilized a randomized crossover design to order the prior exercise and control high-fat meal visits. Visits were separated by four to seven days with the exception of the exercise procedure that occurred the afternoon prior to a HFM visit. A detailed overview of the study protocol and design is depicted in Figure 3.1A. Figure 3.1B further illustrates the timeline of the six total blood draws during the HFM visits. All procedures involving human participants were reviewed and approved by the Iowa State University Institutional Review Board. Written informed consent was received from all participants prior to enrollment.

Participants

Twelve healthy participants, ages 18-35 were recruited to participate in the present study. All participants completed a health history screening and the American College of Sports Medicine guidelines were followed in evaluating whether individuals could safely participate in exercise. Weight and height were measured and used to calculate Body Mass Index (BMI). Body composition was determined using bioelectrical impedance analysis (BIA; InBody 720, GE Healthcare, Madison, WI).

Aerobic capacity test and exercise protocol

Peak aerobic capacity (VO_2 peak) was evaluated on a stationary cycle (Excalibur Sport, Lode BV, Groningen, Netherlands) with a computerized breath-by-breath measurement system (Physiodyne Max-1, AEI Technologies, Pittsburgh, PA). Following a 5-minute warm-up at an intensity of 50W, the aerobic capacity test protocol began at a workload of 80W. The resistance was increased by 30W every 2 minutes until the subject researched exhaustion. Heart rate was monitored continuously and at the conclusion of each stage participants were asked to report their rating of perceived exertion using the Borg scale [19]. In the evening prior to one of the HFM visits, participants completed an acute bout of aerobic exercise. The exercise was performed on the same stationary cycle as the aerobic capacity test and was completed 16-18 hours prior to visit 3. The exercise task was programed at an intensity that corresponded to 65% of VO_2 peak for 45 minutes. Oxygen uptake was measured during the first 15 minutes and for five minutes at the 30-minute time point during exercise to confirm that the bout was being completed at the proper intensity. Venous blood sampling occurred prior to exercise and again within 5 minutes of completing the exercise bout.

Metabolic tracking

Dietary intake was recorded for 72 hours prior to all meal visits. Participants were instructed to follow their normal dietary habits but refrain from alcohol consumption in the 24 hours preceding visits 3 and 4. Diet logs were copied and returned to participants after completion of their first HFM visit and subjects were asked to consume a similar diet for the 72 days prior to their second HFM visit. Research staff visually analyzed the two food diaries for consistency before the second high-fat meal intervention. Physical activity was monitored with a pedometer (Omron, Lake Forest, IL) throughout the experiment and participants maintained usual physical activity. For the control meal visit, subjects were instructed to remain sedentary for the 24 hours before the meal.

High-fat meal

On two separate occasions, participants reported to the laboratory following a 12-14 hour fast. Upon arrival to the laboratory, baseline blood pressure measurements were taken followed by the insertion of a blood sampling line in the participant's forearm vein. After a fasting blood sample was taken, participants were instructed to consume a standardized HFM in fewer than 10 minutes. The meal consisted of two sausage, egg and cheese biscuit sandwiches (Jimmy Dean, Hillshire Brands, Peoria, IL) and 240 ml of 2% chocolate milk (1000 kcal [4.19 kJ], 63 g fat, 27 g saturated fat, 280 mg cholesterol, 78 g carbohydrate, 32 g protein, 1870 mg sodium). Following consumption of the meal, participants had water available to them *ad libitum*. Water volume consumed and time of meal consumption was recorded and matched between visits.

Plasma metabolites

As mentioned previously, six blood samples were collected during visits 3 and 4 by using a forearm venous blood sampling line and heparinized Vacutainer tubes (BD, Franklin Lakes, NJ). Sampling for analysis of plasma TGs, glucose and endotoxin occurred at baseline, 30 minutes, 1 hour, 2 hours, 3 hours and 4 hours following consumption of the high-fat test meal. TGs were assessed using an *in vitro* assay for quantitative determination of products in plasma (Wako Chemicals, Richmond, VA). Plasma glucose concentrations were measured using the glucose oxidase enzymatic assay method (Sigma-Aldrich, St. Louis, MO). Endotoxin was measured using a PyroGene Recombinant Factor C (rFC) endotoxin detection assay (Lonza Walkersville, Inc., Walkersville, MD).

PBMC isolation

PBMCs were isolated from blood drawn during the exercise and HFM visits and all blood was collected into heparinized Vacutainer tubes (BD, Franklin Lakes, NJ). Samples were taken at rest and within five minutes of the 45-minute acute exercise bout, and at baseline and four hour post-meal during the HFM visits. PBMCs were isolated using the Ficoll Paque Plus (GE Healthcare, Uppsala, Sweden) method, aspirated and washed twice with sterile PBS. The approximate yield of PBMCs per sampling time was 8-10 million cells and isolated cells were stored in cell lysis buffer or RNA Lysis Buffer at -80C° for the assessment of protein and gene expression as described below.

Western blotting

Following isolation, PBMCs for Western blotting were lysed with 1% Triton cell lysis buffer (Cell Signaling, Danvers, MA) combined with phosphatase (Sigma-Aldrich, St. Louis,

MO) and protease (Thermo Scientific, Rockford, IL) inhibitors. Protein concentrations were determined using a bicinchoninic acid (BCA) assay compared to a bovine serum albumin (BSA) standard (Thermo Scientific, Rockford, IL). A total protein concentration of 7-28 μ g for each treatment was loaded and run on a 4-15% gradient SDS polyacrylamide gel (Bio-Rad, Hercules, CA), with equal protein loading within each participant. Total protein was transferred from the gel onto a polyvinylidene difluoride membrane (PVDF; EMD Millipore Billerica, MA) and blocked at room temperature for an hour in Tris-buffered saline with 0.05% tween (TBST) and 5% non-fat dry milk. Following the blocking procedure, membranes were incubated in primary antibodies (p-AMPK α ^{T172}, p-AMPK α ^{S485}, p-ACC^{S79}, p-PKD^{S916}, p-GSK3 β ^{S9}, p-NF- κ B p65^{S536}, p-I κ B α ^{S32}, p-ERK^{T202/Y204}, p-JNK^{T183/Y185}, p-p38^{T180/Y182}, Actin, AMPK α , ACC, PKD or I κ B α) at 1:1,000 overnight at 4C° with gentle rocking. Membranes were washed and then incubated with the appropriate secondary antibody at 1:5,000 for one hour at room temperature. All antibodies were purchased from Cell Signaling (Danvers, MA), with the exception of p-ACC, which was purchased from EMD Millipore (Billerica, MA). HRP-linked chemiluminescence solution (Thermo Scientific, Rockford, IL) was placed on the membranes for imaging by a high-sensitivity detection imaging system (Bio-Rad, Hercules, CA). Densitometry was determined using Image J software (National Institutes of Health, Bethesda, MD) and raw data for phosphorylated proteins were normalized to total protein of interest or Actin.

Quantitative PCR

PBMCs for use in quantitative polymerase chain reaction (qPCR) gene expression measurements were stored in TRIzol (Thermo Scientific, Rockford, IL) or RNA Lysis Buffer (Spin Smart, Denville Scientific, Holliston, MA) at -80C° until processed. RNA was isolated

using RNA purification kits (Spin Smart, Denville Scientific, Holliston, MA). RNA was quantified in duplicate with a NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL). A total of 2 ug of RNA for each sample was reverse transcribed using a Maxima First Strand cDNA synthesis kit (Thermo Scientific, Rockford, IL) on a Bio-Rad thermal cycler (10mins at 25C°, 15mins at 50C°, 5mins at 85C°). Resulting cDNA was diluted to 20 ng RNA/reaction for qPCR with iTaq Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) on a Roche LightCycler PCR detection system. Primers for genes of interest are presented in Table 3.1. The $2^{-\Delta\Delta CT}$ method was used for the relative quantification of all gene expression, normalized against the housekeeping genes Actin or GAPDH.

Statistics

Data are presented as mean \pm SEM. Statistical differences for pre- and post-exercise protein measurements were determined using a Student's t-test. A two-way repeated measures analysis of variance (ANOVA) was used for the analysis of pre- and 4hr post-HFM. Pearson's correlation was calculated to determine relationships between variables of interest. Differences were determined statistically significant at a p-value of ≤ 0.05 .

3.3 RESULTS

Anthropometric and physiologic characteristics

Anthropometric and physiologic characteristics of the study population (N = 12) are outlined in Table 3.2. Based on the World Health Organization (WHO) BMI weight classifications, six participants were in the normal range, five were overweight and one was underweight. All participants met the criteria for VO₂ peak and completed the 45 min exercise bout at the appropriate intensity. With the exception of one, all participants

consumed the meal in 10 minutes or less, with a mean time of consumption of 6.8 ± 0.9 min and an average water intake of $0.5L \pm 0.1$.

Acute Exercise PBMC analysis

Western blot images and quantification of AMPK signaling in response to the 45 minute aerobic exercise bout are presented in Figure 3.2A. AMPK activity has been shown to be inhibited by Protein kinase D (PKD, [13]) and Glycogen Synthase Kinase 3 β (GSK3 β , [20]) through the Serine 485 phosphorylation site. Figures 3.2B and 3.2C show that immediately following exercise there were significant decreases in the phosphorylation of both of these AMPK inhibitory kinases (PKD, $p=0.04$ and p-GSK3 β , $p=0.02$). There were no significant differences in the phosphorylation of AMPK at either the T172 or S485 phosphorylation sites. However, there was a significant increase in acetyl-coA carboxylase (ACC) phosphorylation following exercise ($p=0.05$; Figure 3.2D) suggesting greater AMPK activity [21].

Circulating TG, endotoxin and glucose

Postprandial plasma triglyceride, endotoxin and glucose changes with the HFM and are shown in Figure 3.3. Fasting concentrations of TG were significantly lower when aerobic exercise was performed 16-18 hours prior to the HFM ($p=0.02$). TGs were also significantly lower with prior exercise at the 1-hour, 2-hour and 4-hour time points (all $p < 0.05$), accounting for a 16% decrease in TG AUC when compared to the control HFM visit (Figure 3.3B; $p=0.02$). We also noted that fasting TGs were correlated with fasting p-NF- κ B signaling (Figure 3.6A, $p<0.01$) and inversely correlated with post-exercise p-AMPK^{T172} signaling in PBMCs (Figure 3.6B, $p=0.03$). There were no significant differences between

the two trials for fasting or AUC measurements of plasma glucose or endotoxin (Figures 3.3C, D).

Postprandial PBMC protein analysis

The regulation of the AMPK pathway was affected by prior aerobic exercise (Figure 3.4), marked by a main effect of exercise on p-AMPK^{T172} ($p=0.04$). Although we saw maintained p-AMPK^{T172} signaling 4 hours post-meal compared to the control visit, there was no significant difference in p-AMPK^{S485}. For the analysis of ACC phosphorylation, there was an exercise and meal interaction that trended toward significant ($p=0.05$), such that p-ACC decreased in the control visit more than the HFM with prior exercise. Similar to the results from PBMCs obtained before and after exercise, there was a trend toward a main effect of exercise ($p=0.08$) in the phosphorylation of PKD, with decreased p-PKD with prior exercise. To investigate inflammation, we assessed PBMC NF- κ B and MAPK signaling (Figure 3.5). The phosphorylation of I κ B α trended toward a main effect of the HFM ($p=0.07$) and there was a main effect of exercise in p-NF- κ B signaling ($p<0.05$) with lower p-NF- κ B when prior exercise was performed. The HFM induced significant increases in the phosphorylation of extracellular-signal-regulated kinase (ERK) ($p<0.01$) and phosphorylated c-Jun N-terminal kinase (p-JNK 1) signaling trended toward a decrease with prior ($p=0.09$). There were no significant changes seen in the activity of the third major mitogen-activated protein kinase (MAPK), p-p38.

Gene expression

There were no significant differences in the gene expression of Cleaved Caspase-1, IFN γ , IL-1 β , IL-6, IL-10, IL-18, NALP3, NLRP3, PKR, or TNF α between the two experimental conditions or pre- and post-HFM (data not shown).

3.4 DISCUSSION

Acute aerobic exercise has anti-inflammatory effects that reduce the risk of metabolic disease seen with the chronic consumption of HFMs [8]. Peripheral blood mononuclear cells appear to play an integral role in this hyperinflammation and may directly impact the pathophysiology of T2D and CVD through foam cell formation and chemotaxis [15, 22]. The present study examined the mechanisms by which exercise affects inflammation in PBMCs.

AMP-activated protein kinase has recently been recognized for having anti-inflammatory functions in several cell populations [23]. One main mechanism by which AMPK improves inflammation is by directing macrophages away from pro-inflammatory responses through the regulation of I κ B α , GSK3 β , Akt and CREB [24]. Because of this, the down-regulation of AMPK by nutrient excess has been linked to many metabolic diseases seen with chronic low-grade inflammation [25]. Specifically, the down-regulation in PBMCs has implications in adipose tissue macrophage infiltration, the development of insulin resistance [26] and atherosclerosis [27]. Since exercise is a potent physiological activator of AMPK, we analyzed a single, moderate bout of aerobic exercise on AMPK activation in isolated PBMCs. AMPK phosphorylation is known to be more transient than the phosphorylation of other key players in the AMPK signaling cascade [28], which may

explain the lack of significant differences in AMPK phosphorylation. However, we did detect an increase in p-ACC, a downstream protein of AMPK that is regulated through its phosphorylation [21]. Our results show a significant increase in PBMC p-ACC following exercise, reflecting increased AMPK activity and subsequent decreased fatty acid synthesis.

Our data suggests that in response to exercise, increased AMPK activity in PBMCs may be due to a decrease in AMPK inhibitory kinases. We demonstrated that a 45 minute bout of cycling exercise significantly decreased the phosphorylation of PKD and GSK3 β . These findings may demonstrate that acute moderate intensity aerobic exercise decreases AMPK inhibitory kinases in PBMCs of healthy male subjects and may contribute to increased AMPK activity.

Importantly, the phosphorylation of GSK3 β in response to high glucose concentrations may be involved in the migration of monocytes to the vascular wall [29], which is a key factor in the development of atherosclerosis [30]. AICAR, a pharmacological AMPK activator, deactivates GSK3 β , maintaining the integrity of glucose and lipid homeostasis [31]. This presents the possibility that the changes we found in p-GSK3 β are a downstream result of AMPK activation. Altogether these data suggest that exercise may mitigate glucose-induced macrophage infiltration, and subsequent disease risk, through AMPK.

AMPK activation has been shown to inhibit the ability of macrophages and other cell populations from initiating a pro-inflammatory response through the activity of MAPKs [32] and NF- κ B [33]. Previous research suggests that AMPK signaling in PBMCs from healthy men is reduced following one week of a high-fat diet [16]. We observed no significant changes in phosphorylation at the Serine 485 inhibitory site on AMPK, but it is

possible that reductions in AMPK activity require a longer high-fat diet stimulus than a single meal. Similar to our findings in PBMCs before and after acute exercise, we also saw a tendency of lowered p-PKD in the HFM visit with prior exercise compared to control, which may be play a role in maintaining p-AMPK^{T172} signaling.

We investigated circulating endotoxin, or lipopolysaccharide (LPS), as it is one mechanism by which a HFM is thought to contribute to postprandial inflammation [34]. Endotoxin is a component of the cell wall in gram-negative bacteria that when released into circulation can lead to the activation of toll-like receptor 4 (TLR-4), initiating production of pro-inflammatory cytokines and leukocyte immune responses [35]. Although we expected to see a rise in endotoxin following consumption of the HFM, literature suggests that meal endotoxin responses are dose-dependent and differ based on the health status of the population [36] and composition of dietary fat [37]).

MAPKs are serine/threonine kinases that respond to cellular stress stimuli, including cytokines, LPS and extracellular mediator of inflammation, and in response initiate a variety of signaling cascades related to cell proliferation and survival [38]. MAPKs, namely ERK, JNK and p38, are required for cells to initiate pro-inflammatory responses that play a role in the development of T2D and CVD [39]. MAPK activation is characteristically increased in macrophages with consumption of a chronic high-fat diet [40] but when the duration of the high-fat feeding is shortened, MAPK responses are less consistent [16]. Here we report increased ERK1/2 phosphorylation following consumption the HFM which was not impacted by prior exercise. Although prior aerobic exercise had no effect on initiating the stress-signaling pathway, it tended to decrease p-JNK signaling in

PBMCs, suggesting a possible protective mechanism of physical activity and lowered inflammation.

Our results show a trend toward increased postprandial I κ B α phosphorylation in response to the HFM. This indicates that when a HFM is consumed, the body responds by initiating a pro-inflammatory response through I κ B α degradation and its dissociation with NF- κ B. In line with this, we observed significantly lower p-NF- κ B in PBMCs when prior exercise was performed, demonstrating that exercise inhibited both fasting and HFM-induced inflammatory signaling. Chronic exercise training in high-fat diet fed rodent liver, adipose tissue [41] and cardiac muscle [42], as well as human T2D skeletal muscle [43] has been found to inhibit HFD-induced p-NF- κ B. However, to our knowledge this is the first evidence that a single bout of aerobic exercise improves fasting and postprandial NF- κ B signaling in human PBMCs.

Increases in pro-inflammatory gene expression have been reported following a HFM of similar dietary composition used in the current study [44]. However, despite significant changes in ERK and NF- κ B, we were unable to detect any significant genetic changes in fasting and postprandial PBMCs. One potential explanation for our lack of altered gene expression is the absence of increased plasma endotoxin seen in our healthy study population following the HFM. Since endotoxin is a major driver of pro-inflammatory cytokines, without the stimulus it is possible that transcription of these genes is not being initiated to the degree that we would expect. Similarly, although we did find significant differences in p-NF- κ B between the control and prior exercise trials, NF- κ B was not activated in response to the HFM. Since NF- κ B requires nuclear translocation to be

activated [45], it could be possible that NF- κ B did not move into the nucleus and had no impact on gene expression.

In this study we investigated the cellular effects of aerobic exercise on HFM-induced inflammation in human PBMCs. PBMCs encompass many different cell populations, such as lymphocytes, monocytes and dendritic cells, all of which also have their own sub-populations. Responses to a HFM with or without prior aerobic exercise may vary between PBMC cell types [7]. However, leukocyte number and activation in all PBMC subpopulations (monocytes, lymphocytes, granulocytes and total leukocytes) increase to a similar extent 4 hours following consumption of a HFM [46]. Likewise, acute exercise, either performed as peak aerobic effort, 2 hour moderate intensity aerobic exercise or circuit resistance training produced similar increases in total T lymphocyte, neutrophil and monocyte counts immediately following exercise [47]. The use of PBMCs as a mixed population may more accurately reflect the intercellular communication and inflammatory status observed *in vivo* [48]. Although subpopulations of PBMCs seem to be increasing with exercise and a HFM in a similar manner, follow-up investigations could address individual cell populations to more specifically describe the cellular mechanisms behind the investigated phenomena.

The current study was completed with a young, healthy, male population but we did not restrict participation on the basis of physical activity level or dietary habits. For these, and other reasons, there was a large amount of variation seen in the responses to the HFM and aerobic exercise bout. To limit the effects of this variation, we utilized a within-subject research design. Lastly, despite our promising results of exercise on postprandial lipemia and inflammation, these results cannot be widely applied to other populations. Future

research should be conducted to see what effects age, metabolic status, timing or intensity of exercise may have on the regulation of AMPK in PBMCs with regard to HFM-induced inflammation.

In conclusion, a 45 minute bout of moderate aerobic cycling increased AMPK activation in PBMCs from healthy men ages 18-35. This was indicated by an increase in p-ACC and may be due to decreased inhibitory kinases PKD and GSK3 β . Additionally, this single bout of cycling significantly reduced PPL when completed 16-18 hours prior to consumption of a HFM. The administered HFM induced postprandial cellular inflammation, marked by an increase in p-ERK. Some mediators of the inflammatory pathway, such as p-NF- κ B and p-JNK, were attenuated when prior aerobic exercise was performed. Our novel finding that acute aerobic exercise improves fasting and postprandial NF- κ B signaling in human PBMCs contributes support to the anti-inflammatory roles of acute exercise. However, further research is required to identify how these signaling pathways may be altered in the case of metabolic disease and which subpopulations of PBMCs are involved.

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Table 3.1 – Primer sequences used in qPCR analysis.

Gene	Sequence (5' to 3')
Actin	Forward: GATGAGATTGGCATGGCTTT Reverse: GTCACCTTCACCGTTCCAGT
Cleaved Caspase-1	Forward: TTCTGCTCTTCCACACC Reverse: CTACCATCTGGCTGCTC
GAPDH	Forward: GCCATCAACGACCCCTTC Reverse: AGCCCCAGCCTTCTCCA
IFN γ	Forward: GTGGAGACCATCAAGGAAGAC Reverse: CAGGCAGGACAACCATTACT
IL-1 β	Forward: CATGGACAAGCTGAGGAAGA Reverse: TTCAACACGCAGGACAGGTA
IL-6	Forward: GTAGTGAGGAACAAGCCAGAG Reverse: GGACTGCAGGAACCTCCTTAA
IL-10	Forward: GCAGGTGAAGAATGCCTTTAAT Reverse: ATCGTTCACAGAGAAGCTCAG
IL-18	Forward: ACATCCAAGGAAGGCAGCAG Reverse: TTCGTCACTACCTCCCCGG
NALP3	Forward: ACATCCAAGGAAGGCAGCAG Reverse: TTCGTCACTACCTCCCCGG
NLRP3	Forward: CTTCTCTGATGAGGCCCAAG Reverse: GCAGCAAACCTGGAAAGGAAG
PKR	Forward: GATCCTGAGACCAGTGATGATTC Reverse: GGTCCACTTCCTTTCCATAGTC
TNF α	Forward: TCTCAGCCTCTTCTCATTCC Reverse: ACTTGGTGGTTTGCTACGAC

Table 3.2 – Study population characteristics.

Variable	Mean \pm SEM
Age	23.3 \pm 1.6
Mass (kg)	85.8 \pm 9.0
Height (cm)	178.8 \pm 1.5
BMI	24.2 \pm 1.1
% Body Fat	18.6 \pm 2.1
Systolic Blood Pressure	121.0 \pm 2.8
Diastolic Blood Pressure	74.5 \pm 2.5
VO ₂ Peak (ml/kg/min)	44.7 \pm 2.5
Maximum Heart Rate (BPM)	187.4 \pm 3.1
Acute Exercise VO ₂ (% of VO ₂ peak)	64.1 \pm 1.3

Peak aerobic capacity (VO₂ peak) and maximum heart rate refer to measures taken during visit 1 while exercise VO₂ refers to visit 2.

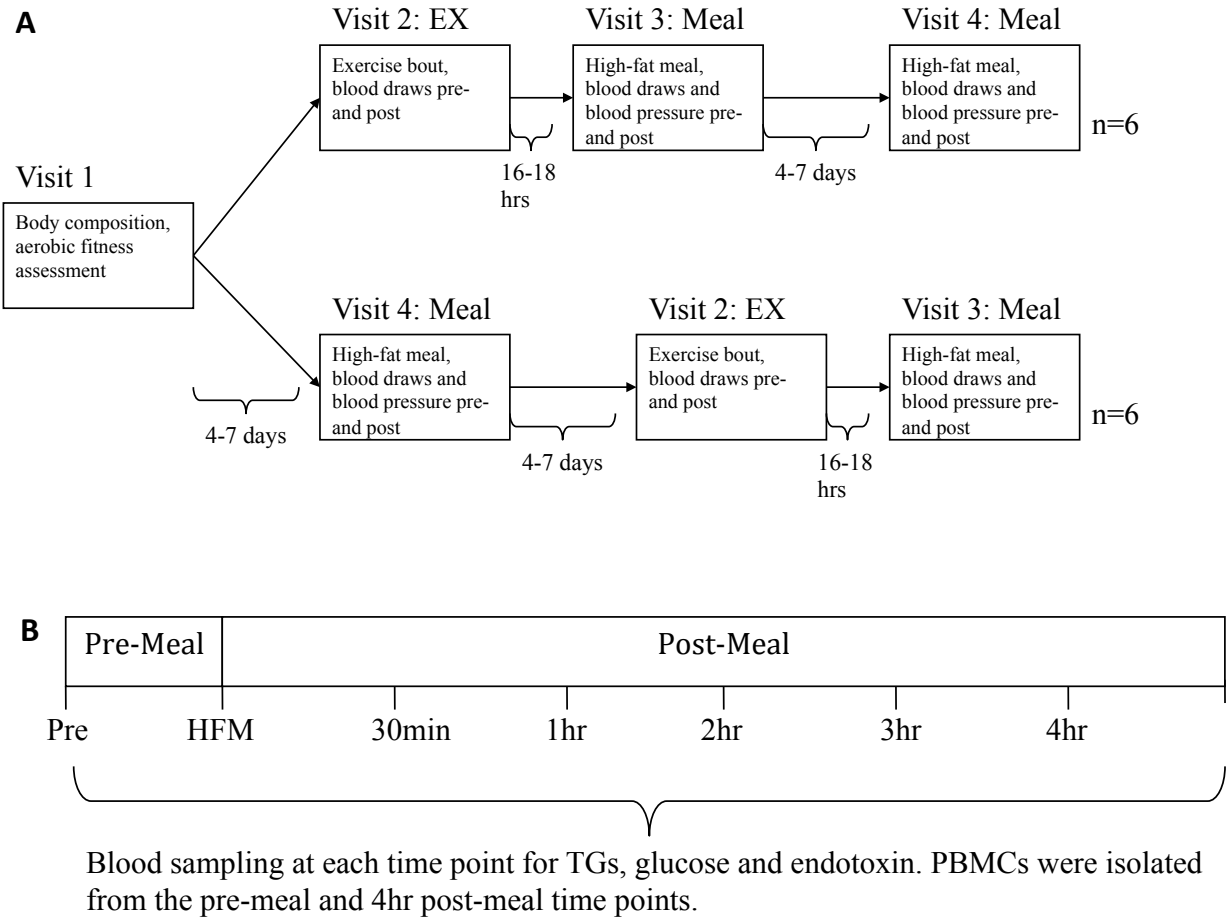


Figure 3.1 – Experimental protocol. A) Overview of experimental procedures for each visit, showing the crossover study design. B) Timeline for high-fat meal (HFM) visits.

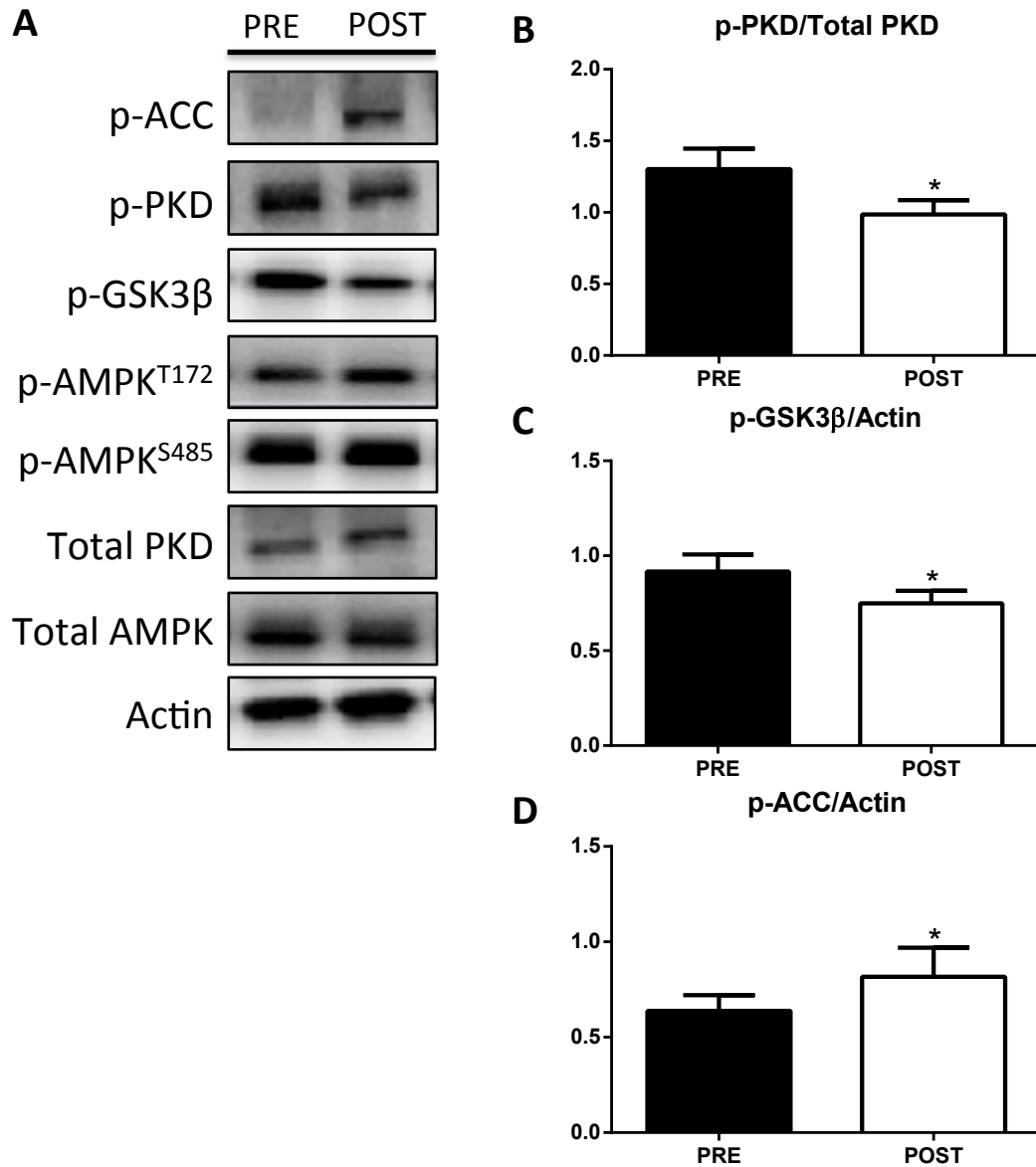


Figure 3.2 – Western blot analysis of PBMCs before and after acute exercise. A) Representative Western blot images of proteins in the AMPK signaling pathway of PBMCs before and after 45 minutes of moderate intensity exercise. Densitometry quantification of data are presented as mean \pm SEM. * $p < 0.05$ versus pre-exercise.

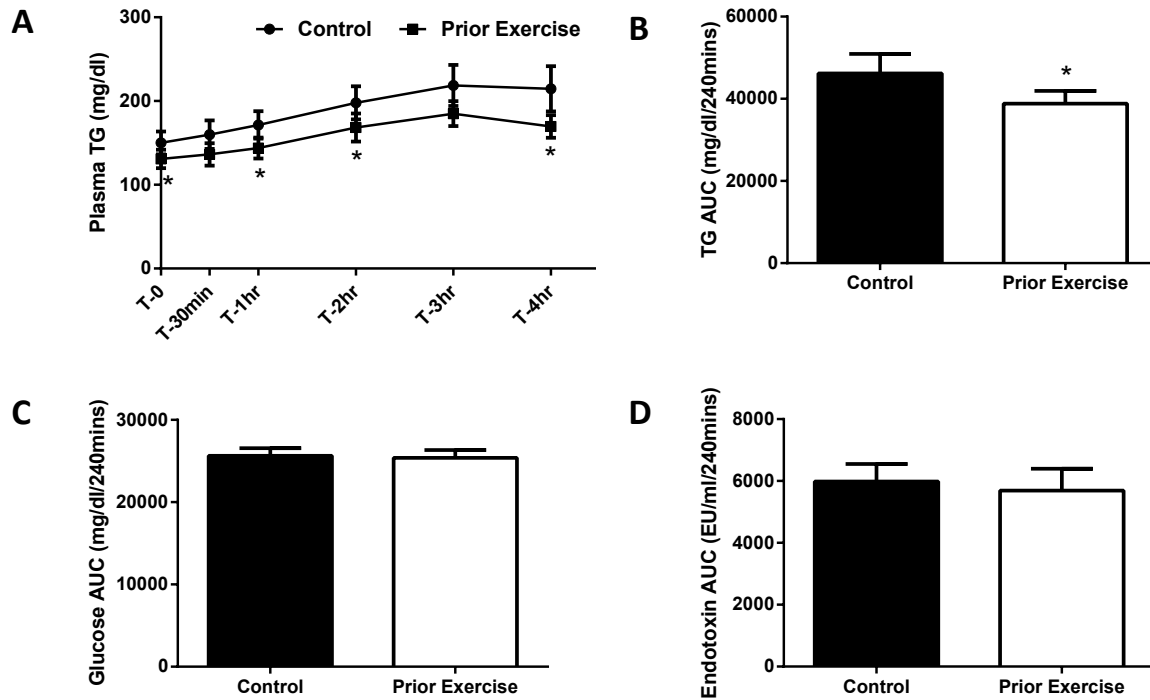


Figure 3.3 – Fasting and postprandial plasma metabolites. A) Repeated measures data represent plasma TGs at baseline and 30 minutes, 1, 2, 3 and 4 hours post-HFM. AUC calculations for the control and prior exercise trial are shown for B) plasma TG, C) plasma glucose and D) plasma endotoxin. Data are presented as mean \pm SEM. * $p < 0.05$ versus control trial.

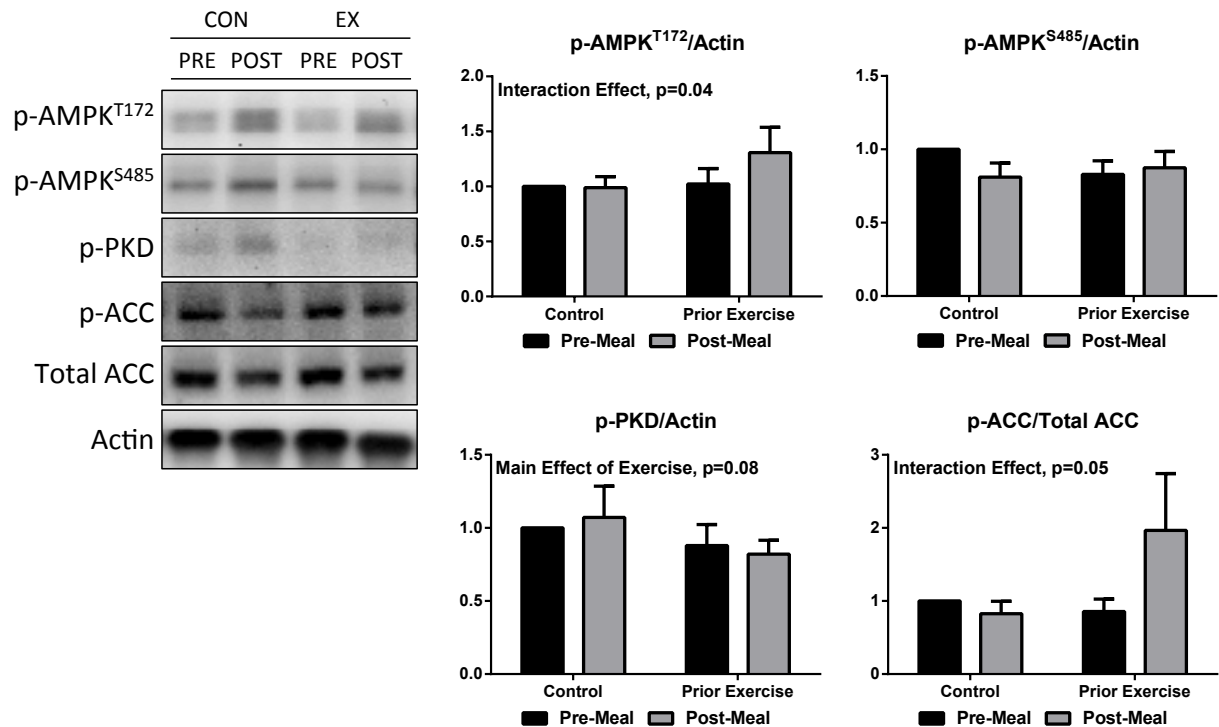


Figure 3.4 – PBMC AMPK signaling following a HFM. Western blot images show AMPK pathway proteins in PBMCs at fasting and 4 hours post-meal with and without prior exercise. Standardized and fold-change results are shown.

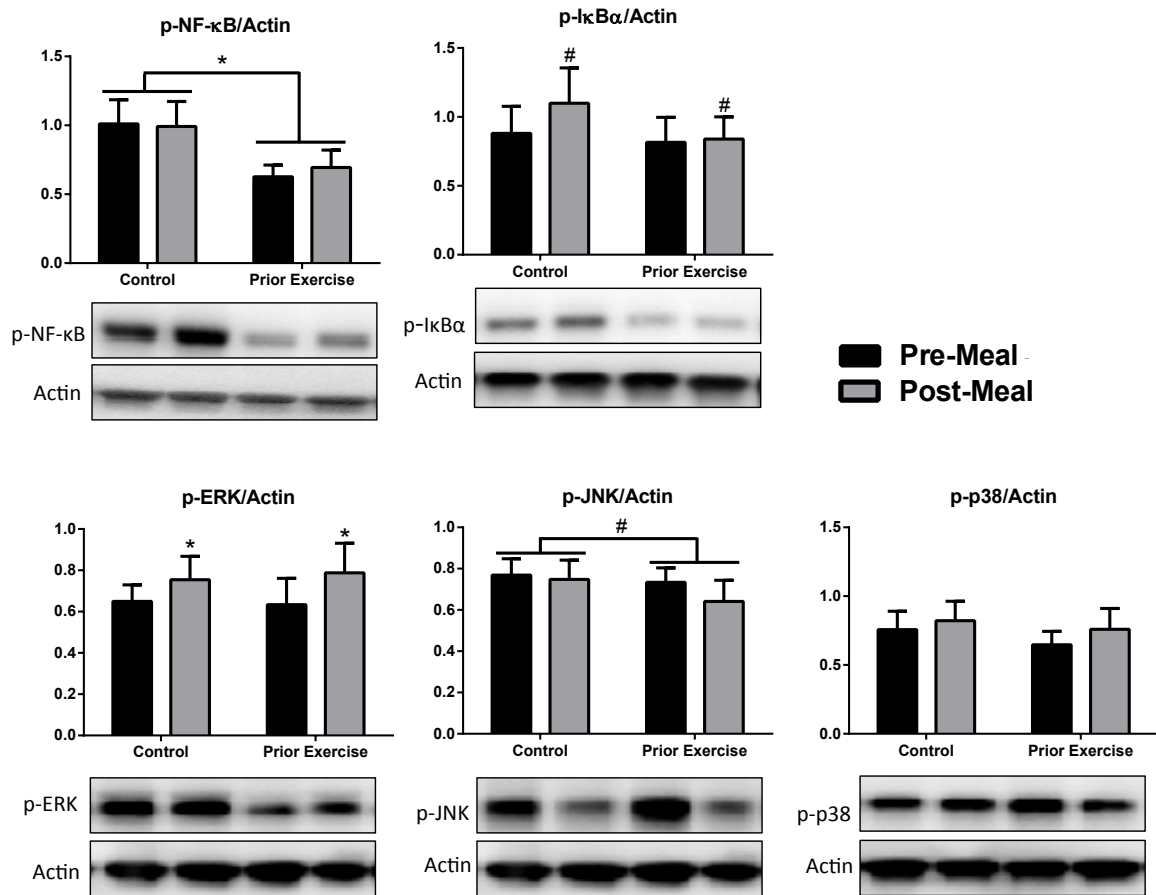


Figure 3.5 – NF-κB and MAPK signaling following a HFM with and without prior exercise. Fold-change results show NF-κB and MAPK signaling in PBMCs at fasting and 4 hours post-HFM with and without prior exercise. All data are presented as mean \pm SEM and a * denotes a significant effect ($p < 0.05$) of either meal or exercise, while # represents a trend toward significance ($p < 0.10$).

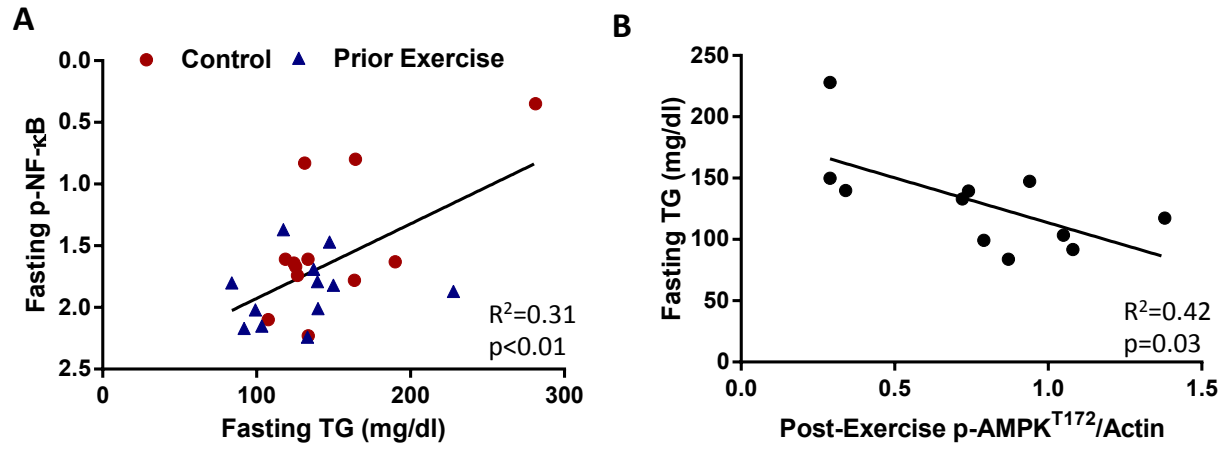


Figure 3.6 – Correlations between TGs, p-NF-κB and p-AMPK signaling. Graphs depict a correlation between A) fasting p-NF-κB and fasting TGs and an inverse correlation between B) post-exercise p-AMPK^{T172} and fasting TG.

CHAPTER 4 – GENERAL CONCLUSIONS

With worldwide rises in obesity, the prevalence of metabolic diseases such as T2D, CVD and metabolic syndrome is increasing. Recent literature has determined that chronic low-grade inflammation is an early and potent contributor in the pathogenesis of these diseases. Similarly, a single HFM has been shown to induce postprandial lipemia and inflammation, also contributing to disease risk. With Westernized diets and a shift toward larger portion sizes and meals with higher fat content, people are spending large amounts of their day in a postprandial state. For these reasons, investigating the postprandial metabolic consequences is very pertinent and of great research interest.

Physical activity is one suggested approach to attenuate the metabolic consequences of consuming high-fat meals. Regular exercise is widely appreciated for lowering chronic disease risk and has many anti-inflammatory effects. This study aimed to determine whether a single bout of moderate aerobic exercise was protective against inflammation and lipemia caused by a high-fat meal and further, to identify potential cellular mechanisms underlying the findings. Peripheral blood mononuclear cells are involved in the immune response and have direct interaction with tissues in the periphery. Based on their integral role in mitigating hyperinflammation, PBMCs were collected from young, healthy, men in order to investigate the research questions.

AMPK signaling is a key pathway in the regulation of metabolism as it promotes cellular adaptation in response to nutrients, hormones and growth factors. AMPK is known as a whole-body energy-sensing enzyme as it samples the energy status of cells and once activated, plays essential roles in many tissues, including skeletal and cardiac muscle, liver and adipose tissue. The down regulation of AMPK by nutrient excess is of great concern

because it has been shown to lead to insulin resistance and the development of other chronic metabolic diseases. More recently, AMPK has been recognized for anti-inflammatory properties, however its regulation in immune cells remains widely unknown. The present study aimed to determine if acute aerobic exercise increased AMPK phosphorylation in PBMCs and if this bout of exercise prevented the down regulation of AMPK induced by the consumption of a HFM.

We found that an acute bout of aerobic exercise increased PBMC AMPK activation as shown by increased ACC phosphorylation. This increased activation is possibly due to a decrease in AMPK inhibitory kinases, PKD and GSK3 β . The exercise intervention also tended to maintain AMPK signaling following a HFM compared to the control visit without prior exercise. These results add support in PBMCs to existing research that shows that exercise is a potent activator of AMPK.

The HFM induced postprandial lipemia and pERK signaling. However, PPL as well as fasting and post-meal measures of inflammation were attenuated when prior moderate intensity aerobic exercise was performed. The largest impact of exercise on postprandial inflammation was seen in PBMC NF- κ B signaling, a transcription factor that is acknowledged as a standard inflammatory marker. Altogether our results show that 45 minutes of acute aerobic exercise is a promising intervention for preventing postprandial lipemia and cellular inflammation through activation of AMPK and decreased NF- κ B signaling.

Our study population was restricted to healthy, young males and therefore cannot be widely applied to other populations. Future research should investigate these same phenomena in women, older adults and those with metabolic diseases such as type T2D.

Additionally, PBMCs are a diverse cell population with many sub-populations of cells. In order to better understand the physiological changes of HFM-induced inflammation with or without prior exercise, specific sub-populations of PBMCs should be investigated.

APPENDIX – INSTITUTIONAL REVIEW BOARD APPROVAL FORM

IOWA STATE UNIVERSITY
OF SCIENCE AND TECHNOLOGY

Institutional Review Board
Office for Responsible Research
Vice President for Research
1138 Pearson Hall
Ames, Iowa 50011-2207
515 294-4500
FAX 515 294-4267

Date: 8/21/2015

To: Dr. Rudy Valentine
243 Forker Building

From: Office for Responsible Research

Title: Effects of prior aerobic exercise on high-fat meal-induced inflammation

IRB ID: 15-336

Approval Date: 8/21/2015

Date for Continuing Review: 6/15/2016

Submission Type: Modification

Review Type: Expedited

The project referenced above has received approval from the Institutional Review Board (IRB) at Iowa State University according to the dates shown above. Please refer to the IRB ID number shown above in all correspondence regarding this study.

To ensure compliance with federal regulations (45 CFR 46 & 21 CFR 56), please be sure to:

- **Use only the approved study materials** in your research, including the recruitment materials and informed consent documents that have the IRB approval stamp.
- **Retain signed informed consent documents for 3 years after the close of the study**, when documented consent is required.
- **Obtain IRB approval prior to implementing any changes** to the study by submitting a Modification Form for Non-Exempt Research or Amendment for Personnel Changes form, as necessary.
- **Immediately inform the IRB of (1) all serious and/or unexpected adverse experiences** involving risks to subjects or others; and (2) **any other unanticipated problems involving risks** to subjects or others.
- **Stop all research activity if IRB approval lapses**, unless continuation is necessary to prevent harm to research participants. Research activity can resume once IRB approval is reestablished.
- **Complete a new continuing review form** at least three to four weeks prior to the **date for continuing review** as noted above to provide sufficient time for the IRB to review and approve continuation of the study. We will send a courtesy reminder as this date approaches.

Please be aware that IRB approval means that you have met the requirements of federal regulations and ISU policies governing human subjects research. **Approval from other entities may also be needed.** For example, access to data from private records (e.g. student, medical, or employment records, etc.) that are protected by FERPA, HIPAA, or other confidentiality policies requires permission from the holders of those records. Similarly, for research conducted in institutions other than ISU (e.g., schools, other colleges or universities, medical facilities, companies, etc.), investigators must obtain permission from the institution(s) as required by their policies. **IRB approval in no way implies or guarantees that permission from these other entities will be granted.**

Upon completion of the project, please submit a Project Closure Form to the Office for Responsible Research, 1138 Pearson Hall, to officially close the project.

Please don't hesitate to contact us if you have questions or concerns at 515-294-4566 or IRB@iastate.edu.